United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program

SOP No.: MDP-MTH-08		Page 1 of 14
Title: Detection of *Shigella* spp. in Fresh Produce by Realtime Polymerase Chain Reaction (rtPCR) and Cultural Isolation and Identification		
Revision: 01	Replaces: none	Effective: 03/01/08

1. Purpose

To provide standard procedures for detecting *Shigella* spp. in preenriched cultures from produce wash samples. To isolate and identify these target organisms from positive samples.

2. Scope

This SOP shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities that may impact the program. This SOP represents minimum MDP requirements and is presented as a general guideline. Each laboratory shall have written procedures that provide specific details concerning how the procedure has been implemented in that laboratory.

3. Principle

The presence of *Shigella* cells in the produce samples will be detected using Realtime PCR (rtPCR). This reaction will specifically amplify the genes coding for the invasion plasmid antigen H (*ipaH*) present in several strains of *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC).

4. Outline of Procedures

5. References

5.1. Pacific Regional Laboratory, Southwest, FDA (PRL SW) Shigella Realtime Assay. A method developed by Wen Lin, PRL SW, FDA dated 02/01/2008.

5.2. Detection of *Shigella* in Fresh Produce by Realtime Multiplex Polymerase Chain Reaction (rt mPCR) and Cultural Identification. A method developed by Division of

Consolidated Laboratories (DCLS), Richmond, VA for Microbiological Data Program (MDP) dated 11/7/2007.

5.3. Bin Kingombe C. I., Cerqueira-Campos, M-L, and J. M. Farber. 2005. Molecular Strategies for the Detection, Identification, and Differentiation between Enteroinvasive *Escherichia coli* and *Shigella* spp. J. Food Protection. 68: 239-245.

5.4. Bacteriological Analytical Manual Online (8th edition, 2001 revision), Chapter 6, *Shigella*. http://www.cfsan.fda.gov/~ebam/bam-6.html, accessed on 02/20/08.

5.5. SmartCycler® II Operator Manual, Cepheid

5.6. E. J. Kontanis and F. A. Reed. 2006. Evaluation of Real-Time PCR Amplification Efficiencies to detect PCR inhibitors. J. Forensic Science. (51):4, 795-804.

5.7. SOP MDP-QA-03, Quality Assurance (QA) Controls

5.8. SOP MDP-SHIP-03, Procedures for Packaging, Shipping, and Archiving Microbiological Cultures

5.9. SOP MDP-DATA-01, Microbiological Record Keeping and Results Reporting

6. Specific Procedures

6.1. Equipment and Materials

6.1.1. Sterile aerosol barrier pipette tips

6.1.2. Cepheid SmartCycler® II

6.1.3. SmartCycler® reaction tubes, 25 uL (Fischer Scientific Cat. No. 900-0085)

6.1.4. SmartCycler® cold blocks and racks

6.1.5. SmartCycler® mini-centrifuge

6.1.6. Microcentrifuge

6.1.7. Micropipettors (2-20 uL, 20-200 uL, 100-1000 uL, or equivalent)

6.1.8. VITEK® system, bioMerieux

6.1.9. GNI+ or GN VITEK identification cards

6.1.10. Vortex Mixer

6.1.11. PCR tubes

6.1.12. Incubator 35-37^{o}C

6.1.13. Incubator 42 ± 2^{o}C

6.1.14. Gloves

6.2. Media and Reagents

6.2.1. Primers and probe

Table 1 Primers/probe sequences

Name	***Sequence (5' → 3')***
SHIG F primer	ACCATGCTCGCAGAGAAACT
SHIG R primer	TACGCTTCAGTACAGCATGC
SHIG probe	[CAL Fluor Red 610]-TGGCGTGTCGGGAGTGACAGC-[BHQ-2]

6.2.1.1.SHIG F and SHIG R primers mixed at a concentration of 10 µM each.

6.2.1.2. SHIG Probe at a concentration of 10 µM.

Note: Probe solutions are light-sensitive and should be stored in Black tubes or otherwise protected from light.

6.2.2. PuReTaq Ready-To-Go PCR™ beads (GE Healthcare, Item no.: 27-9558-01)

6.2.3. DNase-free, RNase-free water (PCR grade water)

6.2.4. MacConkey agar (MA)

6.2.5. Hektoen enteric agar (HE)

6.2.6. Xylose lysine deoxycholate agar (XLD)

6.2.7. Blood agar plates (BA)

6.2.8. Triple Sugar Iron Agar (TSI) slants

6.2.9. Lysine Iron Agar (LIA) slants

6.3. Controls:

6.3.1. Specific strains are listed in SOP MDP-QA-03.

Note: Use MDP-013, Shigella sonnei-GFP as positive control and MDP-017, E. coli as negative control

6.3.2. The following controls shall be run with every batch of samples amplified as applicable. For both DNA and process controls, determine appropriate number of cells (CFU/mL) required for DNA extraction and PCR amplification to avoid control failures. If any of the controls fail to yield a satisfactory result refer to SOP MDP-QA-03.

6.3.2.1. DNA Controls: The DNA extraction and preparation of these controls should be made prior to sample setup. These controls shall be taken through the PCR amplification step.

6.3.2.1.1. Positive MDP-013 DNA control

6.3.2.2. Process controls are taken through the DNA extraction and amplification steps along with samples.

6.3.2.2.1. Negative culture control (refer to SOP MDP-LABOP-02): Use DNA extracted from the negative control culture (MDP-017) used in SOP MDP-04, MDP-MTH-05 and MDP-MTH-07.

6.3.2.2.2. Positive culture control (refer to SOP MDP-LABOP-02): use DNA extracted from the positive control culture (MDP-013)

6.3.2.3. Amplification control: Master mix with primers, probe and PCR grade water (no DNA)

6.4. Safety

6.4.1. *Shigella* spp. are human pathogens and can cause acute intestinal disease (diarrhea, fever, vomiting, and cramps) when ingested orally. Follow the laboratory established biosafety rules. Wear laboratory apron, gloves, and safety glasses during handling of *Shigella* cultures. Wear gloves during handling of PCR reagents, tubes and equipment. Aerosol barrier tips will help to minimize generation of aerosols.

6.5. Amplification

6.5.1. Use the extracted DNA obtained in SOP MDP-LABOP-02. For this procedure test all UPBt preencriched cultures.

6.5.2. 1X Master Mix Preparation Using PuReTaq Ready-To-Go beads (see Table 2)

6.5.2.1. Perform this procedure in a clean room or PCR workstation away from previously amplified material and general microbiological work area.

6.5.2.2. Label and assemble PCR tubes in cooling block or ice bucket.

6.5.2.3. Keep the master mix on cooling block or ice bucket and away from light. Prepare the master mix **without the PCR beads** first. Add beads and gently tap the tube until the beads dissolved. Spin the tube briefly to bring the PCR mix down to the bottom of tube. The PCR master mix should look clear after spin. Prepare sufficient master mix for all sample and control reactions plus at least one additional reaction. See Table 2.

6.5.2.4. Arrange labeled reaction tubes in the SmartCycler® rack and cold block.

6.5.2.5. Add 20 µL PCR master mix to each reaction tube.

6.5.2.6. Add 5 µL template (see section 6.5.1 and SOP MDP-LABOP-02) to the appropriate reaction tube. Close the reaction tubes.

Note: Close the reaction tubes after transferring the master mix. Open one tube at a time to add DNA template and close the tube before proceeding to the next sample. This will help to minimize cross-contamination from aerosol. Spin briefly to bring the PCR mix down to the bottom of the tube.

Table 2. Preparation of PCR master mix

Reagent	*For 1 reaction*	*For (n) + 1 reactions*
Ready-To-Go™ beads	1 bead	1 (n) + 1 bead
DNase-free, RNase-free water (PCR grade water)	16.25 µL	16.25 (n) + 16.25 uL
SHIG F & R primers (each at10 µM)	2.50 µL	2.50 (n) + 2.50 uL
SHIG probe (10 µM)	1.25 µL	1.25 (n) + 1.25 uL

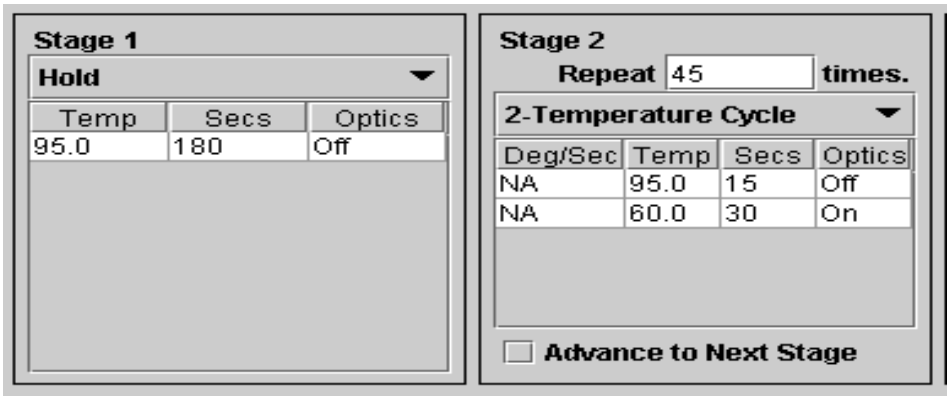
Note: Prior to loading reaction tubes into the SmartCycler® II instrument, the instrument block(s) and the computer must be turned on; the SmartCycler® software must be loaded, and set-up steps 3-5 should be completed. Consult the SmartCycler® II Operator Manual for detailed information on operation of the instrument.

6.5.3 SmartCycler® II Protocol Setup

6.5.3.1 From the main screen, click on the **Define Protocols** icon.

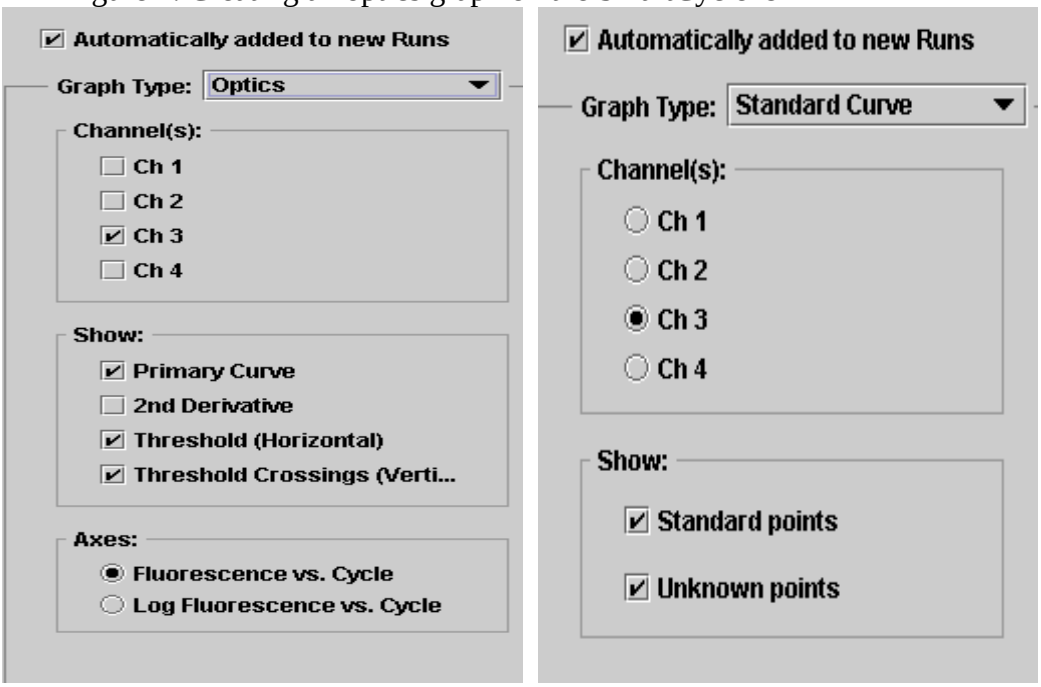
6.5.3.2 Click the **New Protocol** button.
6.5.3.3 Enter a unique **Protocol Name.**
6.5.3.4 Create a two-step thermal cycling protocol according to Figure 1.
6.5.3.5 Click the **Save Protocol** button. Once the protocol has been saved, it is not necessary to create the protocol again for future assay runs.

Figure 1. Thermal cycling parameters for *Shigella* real-time PCR assay.



6.5.4 Defining a Graph on the SmartCycler® II
6.5.4.1 From the main screen, click on the **Define Graphs** icon.
6.5.4.2 Click the **New Graph** button.
6.5.4.3 Enter a unique graph name (for example, **TxR-threshold**) and click **OK.**
6.5.4.4 Check the **automatically added to new Runs** box to add the graph to all new runs. Leave unchecked if the graph should not be added to all runs.
6.5.4.5 Select **Optics** from the **Graph Type** drop-down menu.
6.5.4.6 Check **Ch 3.**
6.5.4.7 From the **Show** menu, check **Primary Curve**, **Threshold (Horizontal)**, and (optional) **Threshold Crossings (Vertical).**
6.5.4.8 From the **Axes** menu, check **Fluorescence vs. Cycle.**
6.5.4.9 Save graph.
6.5.4.10 Refer to left figure in Figure 2 for an example of the graph set-up window.

Figure 2. Creating an optics graph on the SmartCycler® II



6.5.5 Creating a SmartCycler® II Run

6.5.5.1 From the main screen, click on the **Create Run** icon.

6.5.5.2 Assign a unique **Run Name.**

6.5.5.3 Select "FCTC25" from the **Dye Set** drop-down menu.

6.5.5.4 Click **Add/Remove Sites** button to open the **Select Protocols and Sites** window.

6.5.5.4.1 Select the assigned protocol name (6.5.3.3)

6.5.5.4.2 Highlight sites to be used for the assay.

6.5.5.4.3 Click the right pointing arrow to transfer the selected sites to the **Selections** field.

6.5.5.5 Click **OK** to return to the main screen.

6.5.5.6 Load the reaction tubes into the appropriate SmartCycler® II sites. Close covers.

6.5.5.7 Click the **Start Run** button.

6.5.5.8 After the run has been started, the software will automatically switch to the **View Results** screen.

Note: *The user can monitor the temperature and optical data in real time, as well as select graphs,* ***Analysis Settings****, and* ***Sample Type*** *information while the run is in progress. Changes can be made to* ***Analysis Settings****, graphs, and* ***Results Table*** *before, during and after the run is complete; click* ***Update Analysis*** *to see changes. Refer to Figures 3 and 4 below for screen shot examples of* ***Analysis Settings, Results Table*** *and optical data.*

Figure 3. Screen shot showing Analysis Settings and TxR optical data

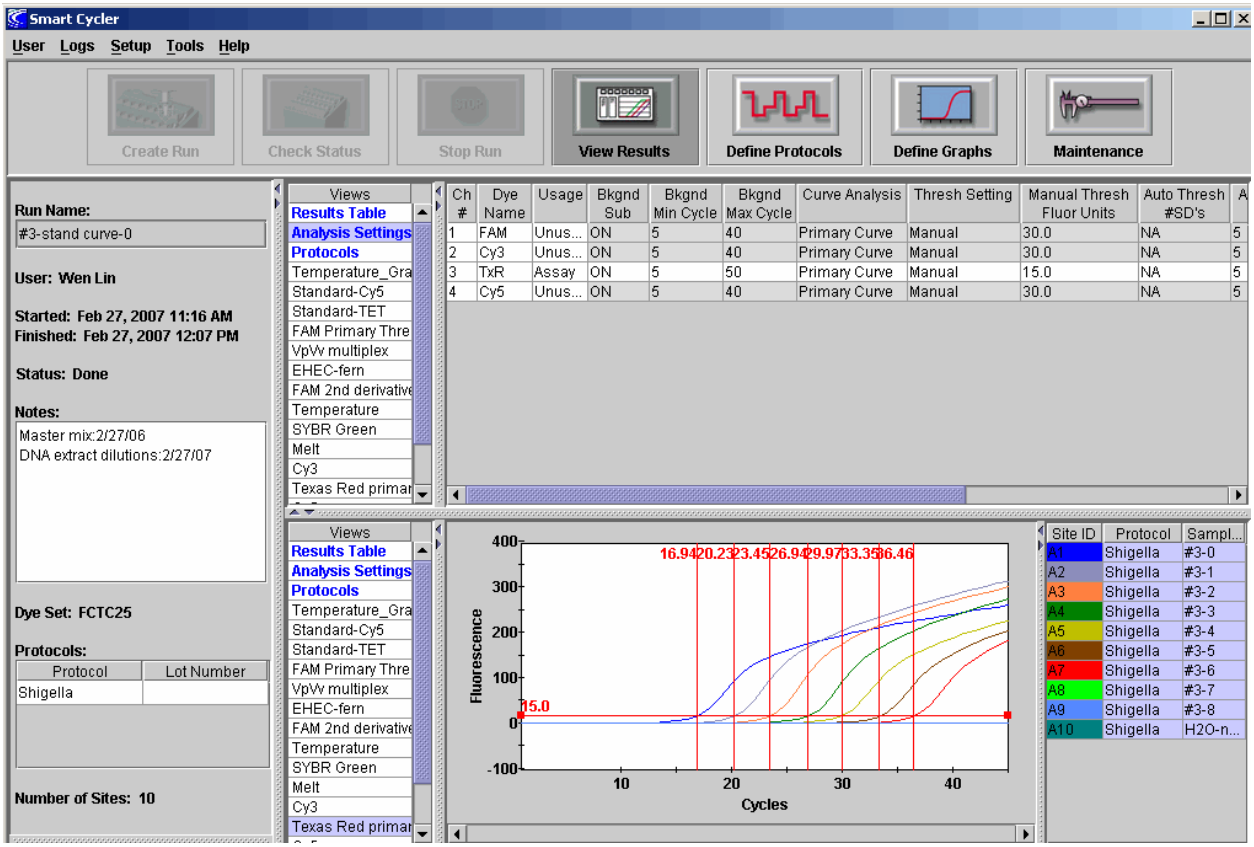
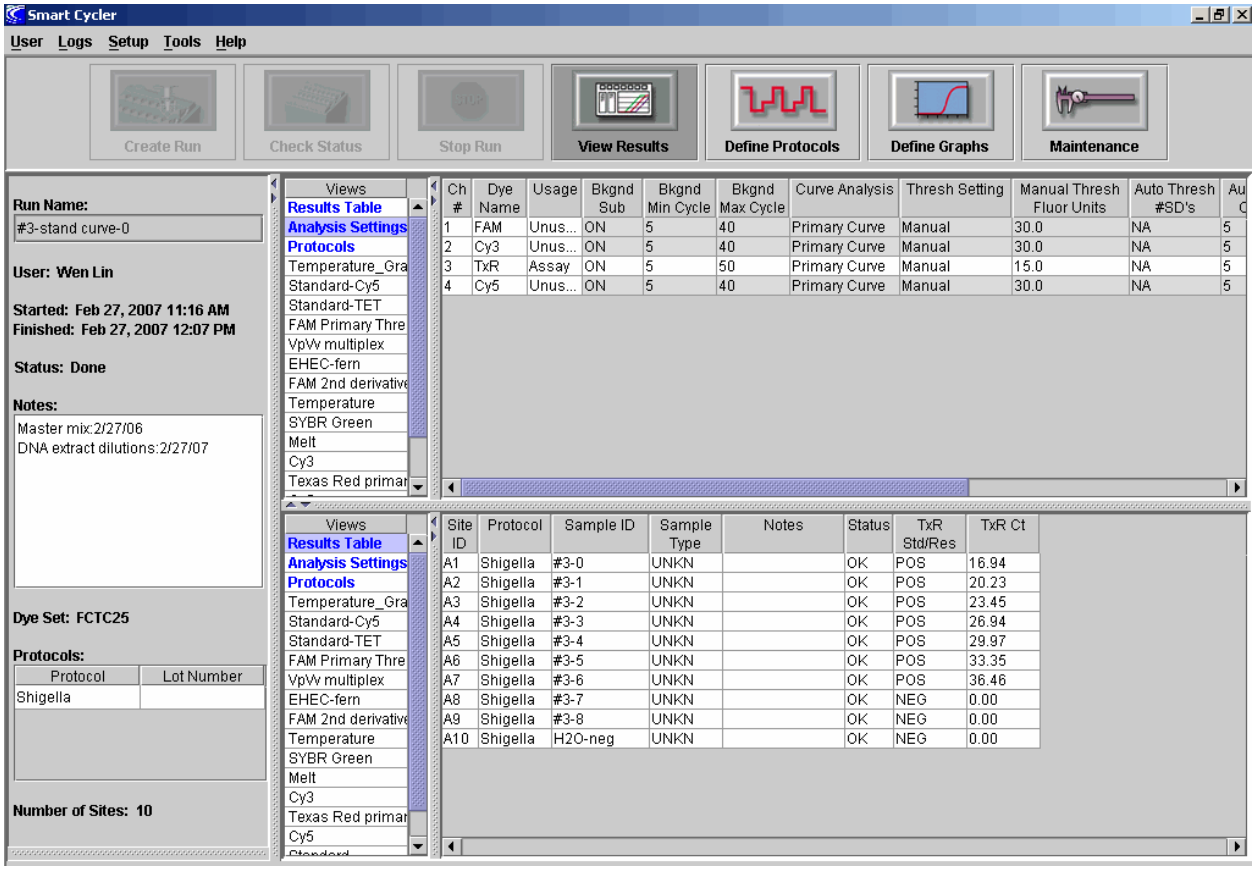


Figure 4. Screen shot showing Analysis Settings and Results Table



6.5.6. Recording Sample Information and Defining Analysis Settings on the SmartCycler® II

6.5.6.1. Click on the View Results icon and select **Results Table** from the **Views** list options. Enter sample information in the **Sample ID** and **Notes** columns as desired.

6.5.6.2. Select **Analysis Settings** from the **Views** list options.

6.5.6.2.1. Update the analysis settings for the **TxR** channel to include the following parameters as needed.

6.5.6.2.2. Turn off channels for FAM, Cy3 and Cy5 dyes by choosing **Unused** from the **Usage** drop-down menu.

Usage	**Assay**
Background subtraction	**ON**
Background Min. Cycle	**5**
Background Max. Cycle	**45**
Curve Analysis	**Primary**
Threshold Setting	**Manual**
Manual Threshold Fluorescence Units	**15.0**
Auto Min. Cycle	**5**
Auto Max. Cycle	**10**
Valid Min. Cycle	**3**
Valid Max. Cycle	**60**
Boxcar Avg. Cycles	**0**

6.6 Detection: Viewing Results on the SmartCycler® II

6.6.1 To view the temperature in real time, click on the **Temperature** graph in the **Views** list.

6.6.2 To view optical data, click on the appropriate graph name (e.g., **TxR-threshold**) in the **Views** list. To add or remove graphs from the **Views** list.

6.6.2.1 Click the **Select Graphs** box at the bottom of the screen.

6.6.2.2 To add a graph, select the desired graph from the **All Graphs** side, and use the arrow button to move to the **Selected Graph** side.

6.6.2.3 To remove a graph, select the graph on the **Selected Graph** side, and use the arrow button to move it to the **All Graphs** side.

6.6.3 View individual curves or combinations of curves by selecting the **Site ID**(s) of interest. (Use the Shift key for contiguous sites or Ctrl key for non-contiguous sites).

6.6.4 When the SmartCycler® II run is completed, click on **Results Table** in the **Views** list.

6.6.4.1 Primary fluorescence curves that cross the threshold will be recorded as **POS** for ***Shigella*** in the **TxR Std/Res** column, and the cycle when the sample crossed the threshold will be recorded in the **TxR Ct** column.

6.6.4.2 Primary fluorescence curves that do not cross the threshold will be recorded as **NEG.**

6.6.5 To create a run report, click the **Report** button at the bottom of the **View Results** screen. Click **Print** to print the report or to create an Adobe PDF file

6.6.6 To print selected curves and their Ct values, place the mouse cursor over the graph, right click and select **Print graph only**.

6.7 Isolation and identification

6.7.1 This section should be followed using the enriched cultures that tested positive for *Shigella* by rtPCR.

6.7.2 Thoroughly mix the positive cultures. Plate 25 uL of each culture onto MacConkey, HE and XLD agar plates. Additional dilutions and subculturing may help to identify *Shigella* colonies among high background microflora.

6.7.3 Incubate plates at 35-37ºC. Check plates at 24 hours and 48 hours for typical (suspicious) colonies (Table 3). Incubation of duplicate set of plates at 42 ± 2^{o}C may reduce background microbial growth.

Note: Use of several selective agar plates, both in type and number, will improve the chance of finding the typical colonies of target isolate on plates.

6.7.4 Transfer 10 typical (and suspicious) colonies (when possible) from each sample to TSI and LIA slants. Incubate the TSI and LIA slants for 24 hours at 35-37ºC. *Shigella* will give the following results: TSI: K/A [alkaline - no color change (red)/acid production - color change to yellow), no H_2S and no gas.
LIA: yellow (acid) butt, indicating acid production and purple slant indicating negative reactions to lysine decarboxylase and lysine deaminase tests; no blackened medium at the apex indicating no hydrogen sulfide reaction.

Note: Shigella is non-motile and doesn't utilize amino acid lysine. Motility and lysine decarboxylase broth tests can be performed in confirming Shigella spp. Refer to FDA BAM Online Chapter 6, Shigella.

6.7.5 Pick five typical colonies and streak them on acceptable media to run on VITEK system using GN or GN+ cards and perform VITEK operation as per manufacturer's instructions.

6.7.6 Choose one typical *Shigella* isolate for archiving and shipping according to SOP MDP-SHIP-03.

Table 3. *Shigella* Characteristics

Media/Condition	**Colony/Culture Characteristics**
MacConkey Agar [with lactose]	White to light pink colonies; colonies small, round w or w/o rough edges; lac-
Hektoen Agar (HE)	Light green colonies to greenish blue without dark centers [the control *S. sonnei*-GFP strain (MDP-013) may display as orange]
XLD	Red colonies [control strain with GFP plasmid may exhibit yellow color when exposed to UV light]
TSI slant	Red slant, yellow butt without gas or hydrogen sulfide production
LIA slant	Purple slant, yellow butt, without blackened apex (no hydrogen sulfide production)
Decarboxylase	Yellow
Lysine decarboxylase	Yellow - negative

6.8 Reporting

6.8.1 A final positive result is defined as an isolated organism identified as *Shigella* spp. by VITEK and showed typical reaction on agar plates.

6.8.2 Preliminary positive (rtPCR) and final result (VITEK and typical reaction on agar plates) need to be reported to MPO using SOP MDP-DATA-01 Attachment 01, Preliminary/Final Results Notification Form.

Disclaimer: Reference to brand names (kits, equipment, media, reagents, etc.) does not constitute endorsement by this agency

SOP No.: MDP-MTH-08		Page 13 of 14
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- Established procedures for detecting *Shigella* spp. in fresh fruit and vegetables using Realtime PCR (rtPCR) assay